# PROTOCOL

The animals were humanely treated with regard to following the standard guidelines for use and care of experimental animals. The research obtained a protocol approval from theUniversity of Ilorin, Ilorin, Nigeria by the Ethical Review Committee (UERC/ASN/2018/1154). This research was conducted in the Animal Holding of the College of Health Sciences, University of Ilorin.

## Grouping and breeding of animals

Bisphenol A and sesame seed oil were procured from Sigma® (CAS –No: 80-05-7), Germany, Also, absolute ethanol and melatonin were purchased from a standard laboratory in Ilorin.

Animals used for the experiment were purchased from an animal breeding farm in Oyo State, Nigeria. Mature female rats weighing 150 ± 10g and male rats weighing 200g ± 20g. Animals were maintained in a controlled environment, with a 12-hour day and night schedule. They had access to water and chow rat-mouse diet freely.

Adult male wistar rats of proven fertility were caged overnight with females in proestrous phase of the cycle.Gestational Day 1 (GD 1) is the day sperm cells aredetected in the vagina.Once pregnancy is confirmed, the pregnant rat was isolated in a cage to stay until parturition, with a total of 12 pregnant females being used for the study. After parturition, female litters were recognized and grouped. These were the second-generation Wistar rats. They had daily subcutaneous injections from post-natal day 0 for four days (PND 0 - 3). The seven groups were administered as follows: I – Normal saline, II – sesame oil and ethanol (vehicle), III - melatonin only (10mg/kg), IV – 25mg/kg BPA, V – 25mg/kg BPA + melatonin (10mg/kg), VI – 50mg/kgBPA, VII – 50mg/kg BPA + melatonin (10mg/kg). All administrations were done in the

morning, and all the groups were allowed to mature till adulthood day 120 ± 3 days, when they were sacrificed in the morning, between 7 a.m. and 9 a.m.

## Sample collection and processing

After administration of BPA and melatonin treatments, the animals were left until day 120 ± 4 days. They were euthanised by administration of 20 mg/kg body weight of ketamine intramuscularly. Following the incision and lateral reflection of the scalp, the skull was promptly exposed with tissue forceps to extract the entire brain. The hypothalamus, situated alongside the pituitary gland at the floor of the sella turcica), was pinpointed, collected, and placed into cryovials preloaded with RNA, then they were subsequently kept frozen in liquid nitrogen tanks. The harvested brains were thenutilized for genetic (RNA) studies. Additionally, the female gonads were also collected; the left ovary for follicular count on histology, which was fixed for 48 hours in 4% paraformaldehyde and the right ovary of each animal was homogenized in 0.25M ice cold phosphate buffer followed by centrifugation of homogenates for enzyme studies. Staining of the ovarian tissues was done in paraffin wax embedded sections according to Canene-Adams, 2013 and Haematoxylin and Eosin staining by the protocols outlined by Fischer *et al.,* 2008. The various follicles (primary, secondary, preantral, antral, and corpora lutea were identified, counted then recorded.

## Hormonal Assay

Blood samples was obtained from the apex of the heart for hormonal analysis using 5 ml needles and syringes. Centrifugation for 15 minutes at 3,000 rpm wasused to separate the serum. The Plasma luteinizing hormone, follicle stimulating hormone, oestrogen, progesterone, testosterone, and Anti-Mullerian Hormone concentration measurements were assayed using the Enzyme-

Linked Immunosorbent Assay (ELISA) technique with ELISA kits purchased from Monobind Inc. Lake Forest, USA, following manufacturer's guide.

## Biochemical assay

Nitric oxide levels were assessed following the method outlined by Montgomery and Dymock in 1961. This involved measuring the levels in an acidic environment, where nitrite, upon formation, was used to create nitrous acid, which reacted with sulfanilamide to form a product. This resulting product was then coupled with N-(1-naphthyl) ethylenediamine, to give an azo dye displaying a vibrant reddish-purple color, quantifiable at 540 nm. The activity of Glutathione peroxidase (GPx) was determined using the method outlined by Paglia and Valentine, 1967. A unit of GPx activity was defined as the enzyme quantity necessary to catalyze the oxidation of 1 nmol NADPH per minute at 25°C.The assessment of Superoxide dismutase (SOD) was carried out following the method detailed by Nishikimi et al. in 1972. The SOD enzyme's capability to impede the phenazine methosulfate-mediated reduction of nitro-blue tetrazolium dye was measured by monitoring absorbance at 560 nm over a 5-minute period at 25°C.

## Quantitative real-time polymerase chain reaction procedure

Euro Gold Tri-Fast solution (Euro Clone) was used to prepare the RNA. The tissue was then pulverized using a tissue homogenizer, followed by DNase treatment on the total RNA samples extracted, such that DNA contamination from the total RNA prepared could be eliminated. Purification (through acid phenol-chloroform), precipitation, and suspension of the RNA in distilled water (dH2O) were then carried out.

The reverse transriptase enzyme (Invitrogen) was used for reverse transcription of total RNA, using M-MLV reverse transcriptase (Invitrogen), which involved retrotranscription of 1 µg of total RNA to quantify mRNA expression in experiments. A gently mixing of the samples

through up and down pipetting and then incubated at 37°C. M-MLV RT was inactivated for 15 minutes at 70°C. cDNA was kept at -20°C.

NanoDrop™ 1000 spectrophotometer was used to measure cDNA and RNA concentrations. Nucleic acid quality was noted via absorbance ratios at 260 nm/280 nm and 260 nm/230 nm. Measurement of the relative amounts of the transcript of a specific gene was done using a qRT-PCR, via a two-step procedure using Sybr green supermix (Biorad)- the amplification reaction and generation of melting curves of the amplicons subsequently. Verification of the melting curve data was through running the PCR product on 2% agarose gel. The efficiency of the Primer was tested in reactions with six serial 1:10 dilutions of cDNA as a template to perform a calibration curve.

## Primers sequence (rattus novergicus)

| | **Primers** | **Sequence** | **Expected Product (bp)** |
|---|---|---|---|
| **1** | **Nuclear receptor 1I3 (NR1I3, CAR)** | | |
| | RT-mCAR-DIR | GCCATGGCTCTCTTCTCTCC | 160 |
| | RT-mCAR-REV | CTAGCAGGCCCATCAGCTTT | |
| | | | |
| **2** | **Androgen receptor (Ar)** | | |
| | RT-mAr-DIR | CAGGGACCACGTTTTACCCA | 229 |
| | RT-mAr-REV | TTTCCGGAGACGACACGATG | |

| | | | |
|---|---|---|---|
| | | | |
| **3** | **Anti-mullerian hormone receptor (Amhr)** | | |
| | mAmh-DIR | CTGGGAGCAAGCCCTGTTAG | 180 |
| | mAmh-REV | GGTTGAAGGGTTAGGGCGAG | |
| | | | |
| **4** | **KISS1 receptor (Kiss1r)** | | |
| | mKiss1r-DIR | GCTAGTCGGGAACTCACTGG | 120 |
| | mKiss1r-REV | ACGCAGCACAGAAGGAAAGT | |
| | | | |
| **5** | **Gonadotropin-releasing hormone 1 (Gnrh1)** | | |
| | mGnrh1-DIR | TGGTATCCCTTTGGCTTTCACA | 192 |
| | mGnrh1-REV | GATCCTCCTCCTTGCCCATC | |
| | | | |
| **6** | **Gonadotropin releasing hormone receptor (Gnrhr)** | | |
| | mGnrhr-DIR | GCCTCAGCCTTGTCTCATGT | 140 |
| | mGnrhr-REV | TATGTTGGGCTTTCCCGGTC | |
| | | | |

## Data analysis

Data collected were analyzed by two-way analysis of variance (ANOVA) and subsequently subjected to Tukey's (HSD) test of multiple comparison using GraphPad Prism v.6 (GraphPad

Software, Inc., La Jolla, CA, USA). Data areexpressed as means ± SEM (standard error of mean). Statistical significance was taken asP value less than 0.05 ($p<0.05$).